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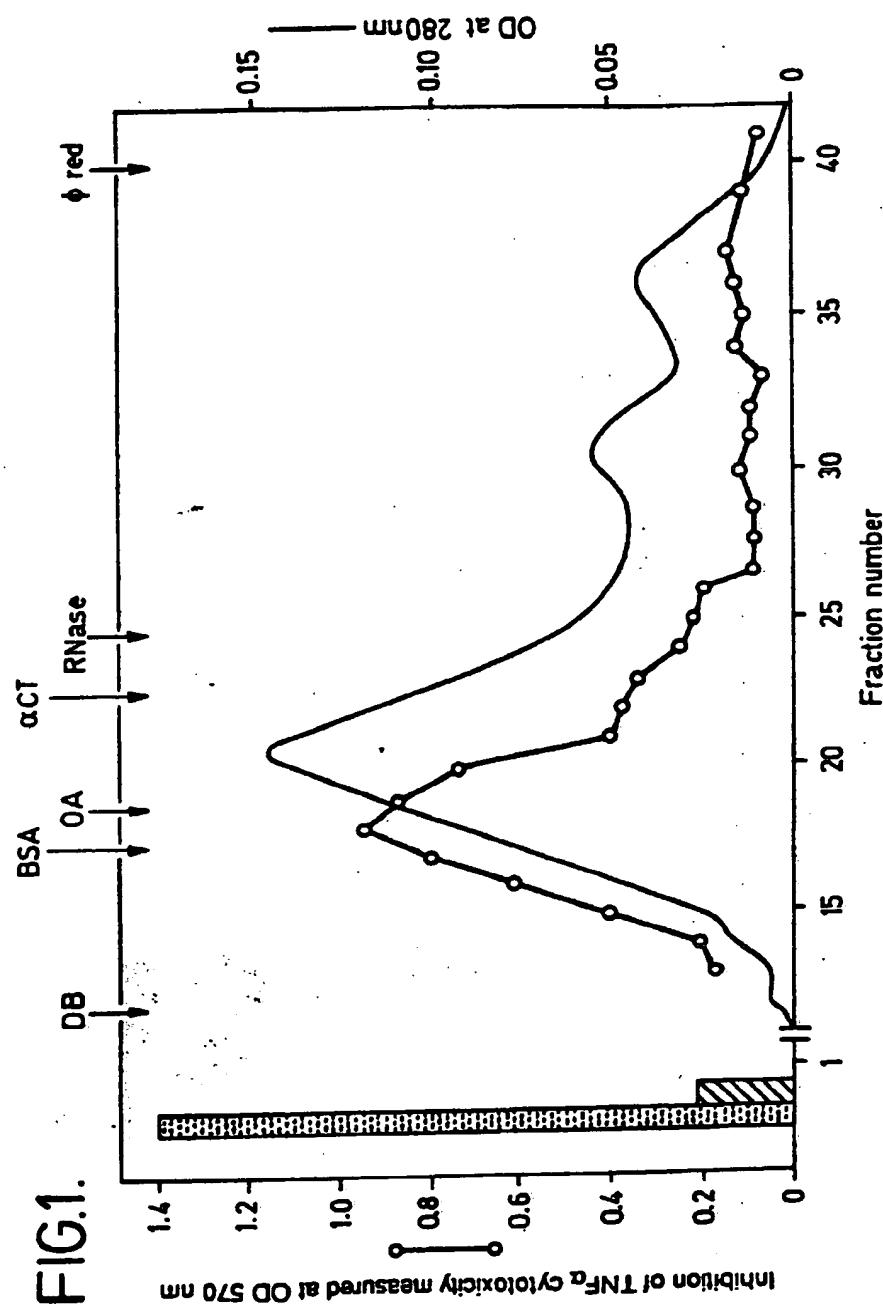
(54) TNF-alpha Inhibitors.

(57) A protein having a selective tumour necrosis factor (TNF)- α inhibitory activity, but which does not block other proteins, particularly IL-1, is prepared by extraction from urine of febrile patients.

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1 / 5



2218101

2 / 5

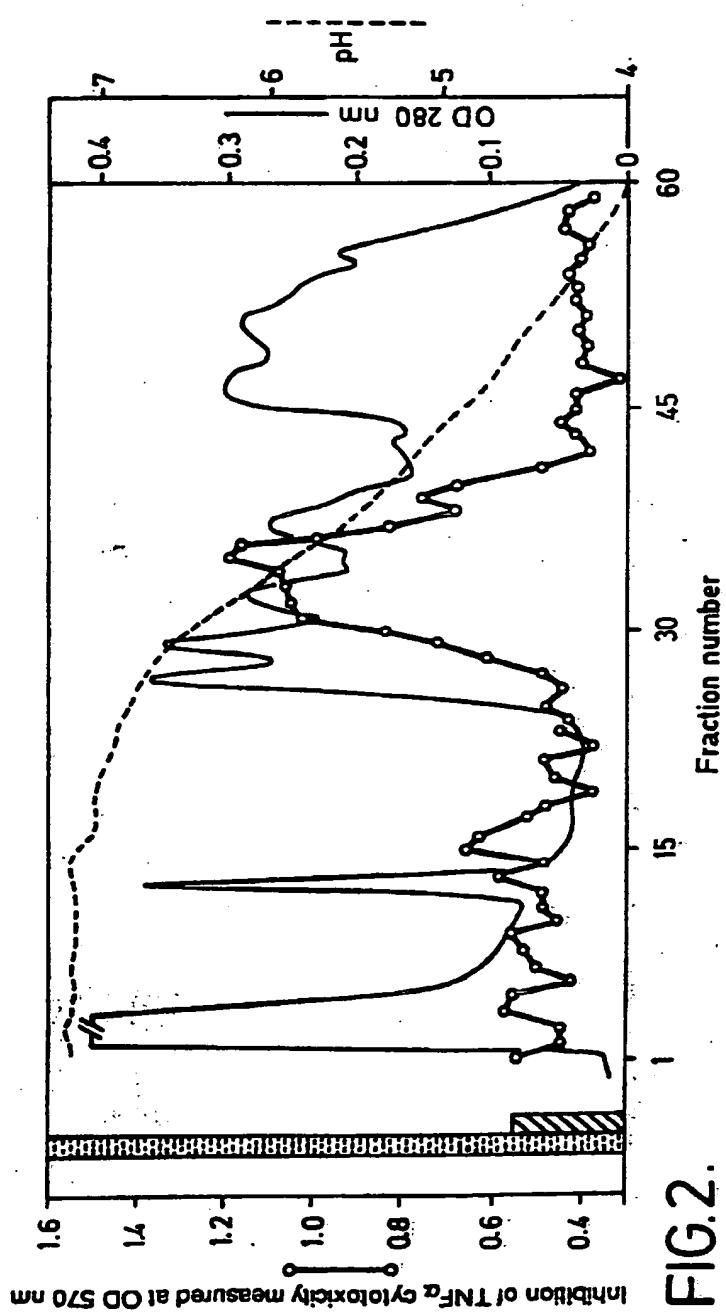


FIG. 2.

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3 / 5

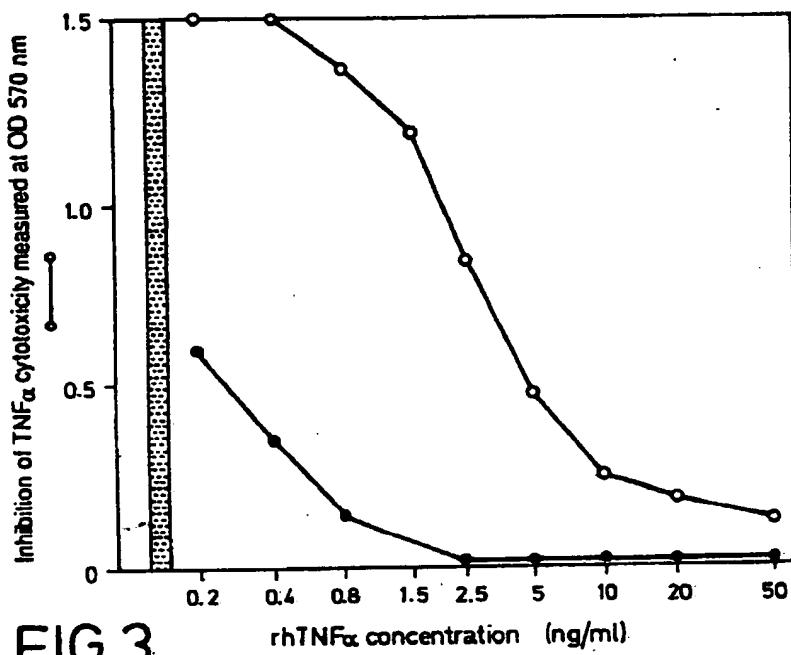


FIG.3.

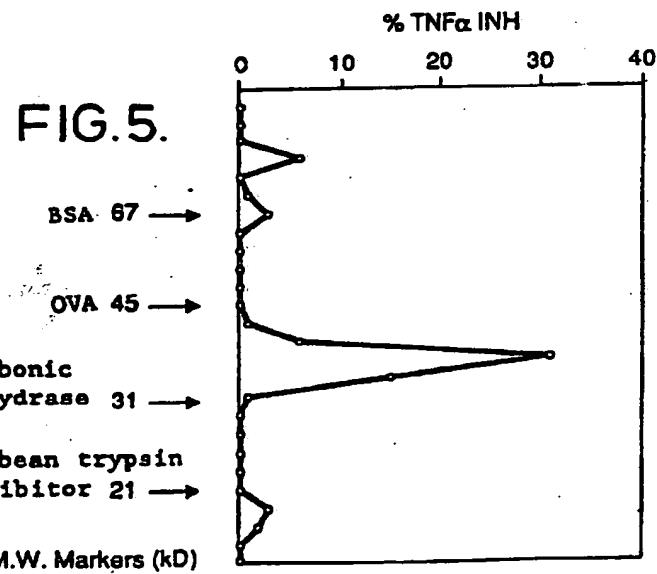


FIG.5.

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4 / 5

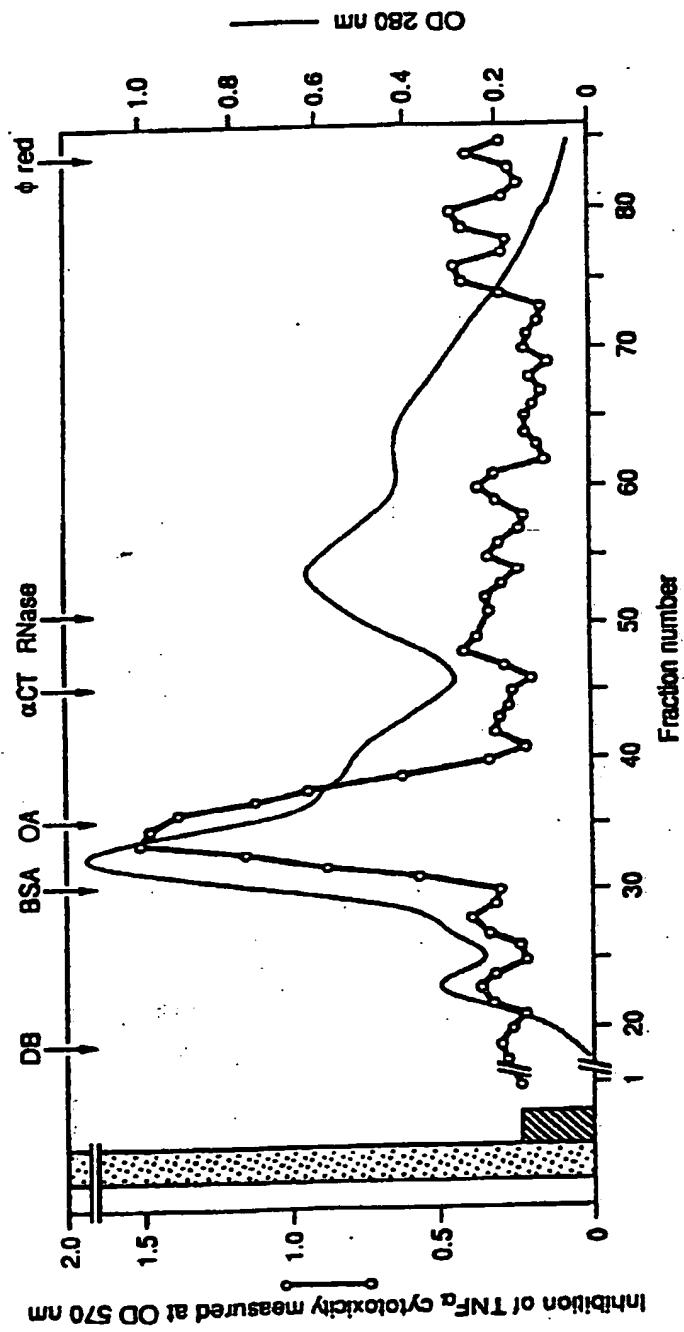


FIG.4.

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5 / 5

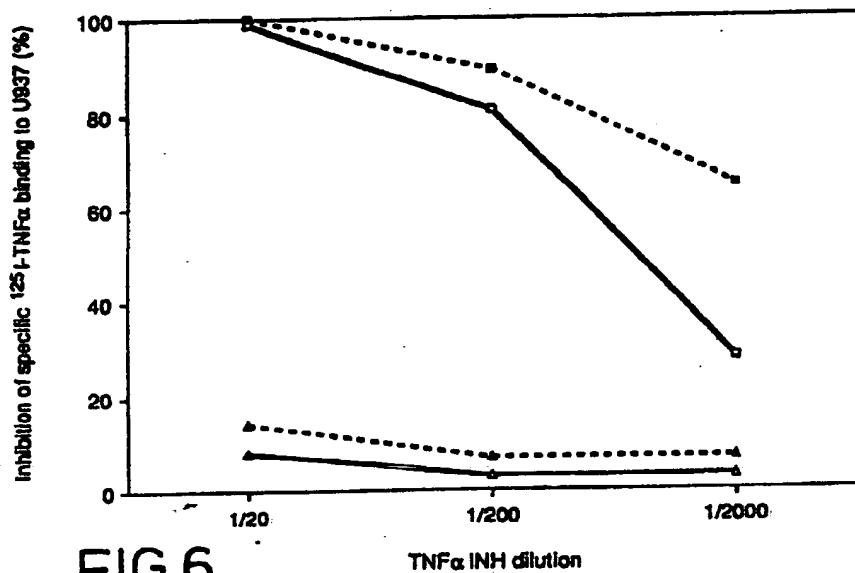


FIG.6.

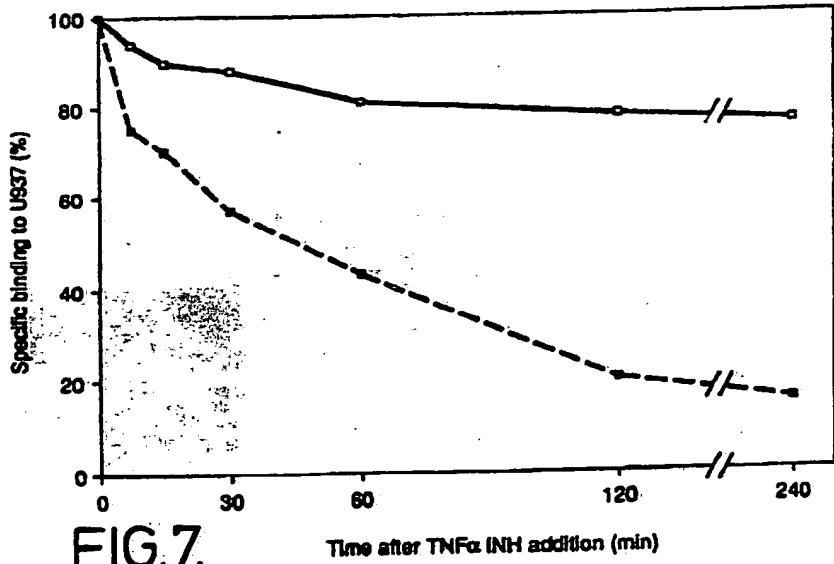


FIG.7.

BIOLOGICALLY ACTIVE PROTEINS

The present invention relates to a novel protein having an inhibitory effect against Tumour Necrosis Factor α -mediated activity, 5 to the isolation and purification of such a protein from natural sources, to its preparation by DNA manipulation and to the use of such a protein in the treatment of conditions associated with excessive or unregulated TNF α production.

Tumour necrosis factor (TNF) is an activity embodied by a 10 family of at least two proteins, α and β , which are cytotoxic for tumour cells and inhibit their growth in culture [E. Carswell et. al. "An endotoxin-induced serum factor that causes necrosis of tumours", Proc. Natl. Acad. Sci. USA, 72, p3666 (1975)]. Tumour necrosis factor α (TNF α), also termed "cachectin", is mainly produced by cells 15 of the monocyte/macrophage lineage in response to "stress" signals which accompany invasive stimuli such as bacteria, viruses, tumours and other toxins. TNF β , commonly termed "lymphotoxin", is mainly produced by lymphoid cells. TNF β has many activities similar to those of TNF α , but it appears to be less potent although this may be as a result of 20 difficulties in preparing pure TNF β .

TNF α mediates and participates in a wide range of biological activities [B. Beutler et. al., "Identity of tumour necrosis factor and the macrophage-secreted factor cachectin", Nature, 316, p552 (1985)] sharing several of them with interleukin 1 (IL-1) [J. Le et. al., "Tumour necrosis factor and interleukin 1 : cytokines with multiple overlapping biological activities", Laboratory Invest., 56, p234 (1987)]. Elevated levels of TNF α induced by, for example, tumour

cells may lead to weight loss and cachexia and TNF α has also been implicated as a principal mediator of endotoxic shock (septic shock) which can be fatal. Other biological effects of TNF α include hypotension, fever (induced by stimulation of hypothalamic 5 prostoglandin E₂ (PGE₂) synthesis), coagulopathy (induced by stimulation of vascular endothelial cells which release, for example, tissue factor) and tissue destruction (induced by, for example, stimulation of a series of proteinases, including collagenase production by dermal fibroblasts and synovial cells) [C. Dinarello 10 et. al., "Tumour necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1", J. Exp. Med., 163, p1433 (1986); J. Dayer et. al., "Cachectin/tumour necrosis factor stimulate collagenase and prostaglandin E₂ production by human dermal fibroblasts and synovial cells", J. Exp. Med., 162, p2163 (1985)]. 15 There exists, therefore, a need to develop a cachectin/TNF α inhibitor which prevents endotoxic shock, cachexia and the other deleterious effects described above. It has been shown that passive immunisation of animals against cachectin can prevent 20 endotoxin-induced death, mediated by TNF α antibodies [B. Beutler et. al., Nature, 316, supra].

We have now identified a novel protein which has a potent inhibitory effect against TNF α -mediated activities without significant concomitant inhibition of IL-1-mediated activity. The protein is hereinafter identified as Tumour Necrosis Factor α Inhibitor (TNF α INH). 25

Thus in one aspect of the invention, we provide a protein which selectively inhibits tumour necrosis factor α -mediated activity.

As used herein, selective inhibition as shown by the inhibitor of the invention is identified as the ability to block TNF-mediated activity while lacking the ability to block other proteins which have in common with TNF certain but not all of the biological activities of 5 TNF, such as IL-1.

Preferably, the tumour necrosis factor α inhibitor of the invention is in a substantially homogeneous form, being substantially free from major contaminants and/or substantially free from other proteinaceous material.

10 The tumour necrosis factor α inhibitor according to the invention has been found to have one or more of the following characteristics:

- (a) a molecular weight in the range 40 to 60 kDa, determined by molecular sieve chromatography;
- (b) an iso-electric point (pI) in the range 5.5 to 6.1, 15 determined by chromatofocussing;
- (c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D, as described by G. Nedwin *et. al.* "Effects of interleukin 2, interferon- γ and mitogens on the production of tumour necrosis factors α and β ", J. Immunol., 135, p2492 (1985). This inhibition can be overcome by further addition of TNF α , indicating that the inhibition is competitive. The inhibitor is also an inhibitor of TNF β activity, although inhibition of TNF α in this assay is more efficient than that 20 of TNF β ;
- (d) inhibition of TNF-induced PGE₂ release from human 25 fibroblasts and synovial cells;

(e) the inhibitor interferes with the binding of TNF α to U937 cells (a monocytic tumour line) as evidenced by inhibition of binding of radiolabelled TNF α (125 I-TNF α);

5 (f) the dissociation of a pre-formed TNF α : U937 cell complex is promoted by the inhibitor in a temperature dependent manner;

(g) the inhibitor does not degrade TNF by proteolytic cleavage;

10 (h) the inhibitor does not inhibit IL-1 receptor-binding activity e.g. the binding of radiolabelled IL-1 (125 I-IL-1 α) to the murine thymoma subline EL4-6.1.

We have found that the protein of the invention when further purified has a molecular weight of about 33000 daltons as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

15 There is thus provided as a further or alternative aspect, a protein which selectively inhibits TNF α -mediated activity which has one or more of the following characteristics :

(a) a molecular weight of about 33 kDa, determined by SDS PAGE;

20 (b) an iso-electric point (pI) in the range 5.5 to 6.1, determined by chromatofocusing;

(c) inhibition of the standard TNF assay of differential cytotoxicity for murine L929 cells treated with actinomycin D, as described by G. Nedwin *et. al.* "Effects of interleukin 2, interferon- γ and mitogens on the production of tumour necrosis factors α and β ", J. Immunol., 135, p2492 (1985). This inhibition can be overcome by further addition of TNF α , indicating that the inhibition is

competitive. The inhibitor is also an inhibitor of TNF β activity, although inhibition of TNF α in this assay is more efficient than that of TNF β ;

- (d) inhibition of TNF-induced PGE₂ release from human
5 fibroblasts and synovial cells;
- (e) the inhibitor interferes with the binding of TNF α to U937 cells (a monocytic tumour line) as evidenced by inhibition of binding of radiolabelled TNF α (¹²⁵I-TNF α);
- (f) the dissociation of a pre-formed TNF α : U937 cell complex
10 is promoted by the inhibitor in a temperature dependent manner;
- (g) the inhibitor does not degrade TNF by proteolytic cleavage;
- (h) the inhibitor does not inhibit IL-1 receptor-binding activity e.g. the binding of radiolabelled IL-1 (¹²⁵I-IL-1 α) to the
15 murine thymoma subline EL4-6.1.

Preferably the TNF α INH of the present invention has both of the characteristics (a) and (b) and one or more of the characteristics (c) to (h).

In particular, the TNF α INH of the present invention has all of
20 the characteristics (a) to (h).

The protein of the invention has an amino terminal amino acid sequence as follows :

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-

Pro-Gln-Cys-Asn-Ser-Ile

25 It is further believed that the next three amino acids provide a glycosylation site and that the sequence thus continues Asn-Ser-Thr-Lys.

It will be appreciated that a TNF α inhibitor according to the invention will comprise an amino acid sequence substantially corresponding to the sequence of native TNF α INH and containing an amino terminal sequence substantially identical to that described above. The sequence of a TNF α inhibitor according to the invention will thus be identical to the sequence of native TNF α INH or contain one or more deletions, substitutions, insertions, inversions or additions of allelic origin or otherwise, the resulting sequence will have at least 80% and preferably 90%, homology with the sequence of native TNF α INH and retain essentially the same biological properties of the protein.

The TNF α inhibitor of the invention has been demonstrated to be proteinaceous in that it is inactivated by heating in a time and temperature dependent manner and is destroyed by treatment with trypsin or pronase.

The TNF α INH of the invention has also been shown to be a glycoprotein since treatment with the enzyme Endoglycosidase F reduces the molecular weight by 7 to 8 kDa.

In a further or alternative aspect of the invention there is thus provided a TNF α inhibitor as defined herein, but which is in a substantially unglycosylated state.

The inhibitors of the invention are of interest in the treatment of conditions in which it is desirable to inhibit TNF α activity, for example, those which arise from the effects of TNF α such as weight loss, shock, cachexia and chronic local inflammation, rheumatoid arthritis, disseminated intravascular coagulation and nephritis.

- 7 -

There is thus provided as a further aspect of the invention a
TNF α inhibitor as herein defined or a pharmaceutically acceptable
derivative thereof for use as an active therapeutic agent, in
particular, in the treatment of conditions associated with excessive
5 or unregulated TNF α production.

In a further or alternative aspect of the invention there is
provided a method for the treatment of conditions associated with
excessive or unregulated TNF α production in a mammal including man
comprising administration of an effective amount of a TNF α inhibitor
10 as herein defined or a pharmaceutically acceptable derivative
thereof.

There is also provided in a further or alternative aspect of the
invention use of a TNF α inhibitor as herein defined or a
pharmaceutically acceptable derivative thereof for the manufacture of
15 a medicament for the treatment of conditions associated with excessive
or unregulated TNF α production.

It will be appreciated by those skilled in the art that reference
herein to treatment extends to prophylaxis as well as the treatment of
established conditions or symptoms.

20 It will be further appreciated that the amount of TNF α inhibitor
of the invention required for use in treatment will vary not only with
the route of administration but also with the nature of the condition
being treated and the age and condition of the patient and will be
ultimately at the discretion of the attendant physician or
25 veterinarian. In general however, a suitable dose will be in the
range of from about 5.0 to 500 μ g per kilogram of bodyweight per day,

for example, in the range 30 to 300 μ g/kg/day, preferably in the range 50 to 150 μ g/kg/day.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day.

While it may be possible that, for use in therapy, a TNF α inhibitor of the invention may be administered as the raw protein it is preferable to present the active protein as a pharmaceutical formulation.

10 The invention further provides a pharmaceutical formulation comprising a TNF α inhibitor as herein defined or a pharmaceutically acceptable derivative thereof together with one or more pharmaceutically acceptable carriers thereof and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be 15 'acceptable' in the sense of being compatible with the ingredients of the formulation and not deleterious to the recipient thereof.

20 The inhibitors according to the invention may therefore be formulated for parenteral administration (e.g. by injection, for example bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusions or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions or solutions in aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the 25 active ingredient may be in powder form obtained by aseptic isolation of sterile solid or by lyophilisation from solution, for constitution

with a suitable vehicle, e.g. sterile, pyrogen-free water, before use.

The TNF α inhibitor of the invention may also be used in combination with other therapeutic agents, for example, other cytokines or inhibitors thereof.

5 The invention thus provides, in a further aspect, a combination comprising a TNF α inhibitor as herein defined or a pharmaceutically acceptable derivative thereof together with another therapeutically active agent, for example, other cytokines or inhibitors thereof.

10 The proteins of the invention may be prepared by purification from natural sources and, where appropriate followed by chemical modification, or they may be prepared by conventional methods known in the art for the preparation of proteins, for example, by recombinant DNA techniques.

15 According to a further aspect of the present invention, there is provided a process for producing the tumour necrosis factor α inhibitor of the invention by purification from natural sources, particularly the urine of human febrile patients. Such purification, for example, comprises the steps of concentrating the crude urine of 20 febrile human patients, precipitating crude TNF α INH from the urine and fractionating the TNF α INH from the other proteins of this precipitate by one or more of, for example, ion exchange column chromatography, gel filtration chromatography, hydrophobicity chromatography, immunoabsorption and affinity chromatography on immobilized TNF α .

25 The tumour necrosis factor α inhibitor of the invention is also obtainable from macrophages containing human tissue, for example,

lung lavages and extracts of human liver, from which it may be obtained by standard purification techniques such as those described above.

Natural and recombinant TNF α INH produced according to the procedures described herein may be purified through a series of steps as listed above. After each of the purification steps, the presence and purity of the TNF α INH may be measured in an assay of cytotoxicity in the presence of actinomycin D (acti D) using a TNF-susceptible cell line L929, as described by G. Nedwin et al., J. Immunol., 135, loc. 10 cit.

In a preferred embodiment of the process the TNF α INH is initially isolated from untreated urine collected from febrile human patients ($>38.5^{\circ}\text{C}$) devoid of urinary infections using a standard concentration technique, for example, ultrafiltration. A crude fraction may then be precipitated from the crude urine using ammonium sulphate e.g. by addition of ammonium sulphate to a concentration of 80% (w/v) at 4°C with stirring. Preferably the ammonium sulphate may be added in a stepwise manner and material precipitated at lower concentrations e.g. at 40% (w/v) discarded. The ammonium sulphate may 15 be removed by dialysis and the resulting fraction purified to separate the TNF α INH from other proteins by a variety of chromatographic methods. 20

Thus, the TNF α INH concentrate may be purified by ion-exchange chromatography which separates proteins according to their differences 25 in electrical charge, which is a reflection of the acid/base properties of the proteins. Suitable materials for anion-exchange chromatography include aminoethylcellulose derivatives, for example,

quaternary-aminoethylcellulose (QAE-cellulose) or diethyleaminoethyl-cellulose (DEAE-cellulose) which are widely commercially available. The anion-exchange column should be equilibrated prior to applying the concentrate using a suitable buffer, such as Tris-HCl, optionally containing a chelating agent such as EDTA. Bound material may be eluted from the column using a salt solution (for example, 0.8M sodium chloride made up with the equilibration buffer).

The pooled active fractions from the anion-exchange chromatography. Suitable materials for cation-exchange chromatography 10 include derivatives of cellulose such as carboxymethyl (CM) cellulose or Sulphopropyl Sepharose (Pharmacia, Uppsala, Sweden). The column should be equilibrated with a suitable buffer, such as sodium acetate and bound material may be eluted with the equilibration buffer containing, for example, 0.5M sodium chloride.

15 The pooled active fractions are further purified by affinity chromatography on bound recombinant human TNF α (rhTNF α), coupled to a suitable matrix, for example, Mini-Leak Agarose (Kem En Tec, Biotechnology Corp., Denmark). The column should be buffered using, for instance, a phosphate buffer (e.g. 0.8M potassium phosphate pH 8.6). Active groups not bound to rhTNF α should be blocked using 20 ethanalamine-HCl pH 8.5 buffer. The column should be equilibrated with a suitable buffer, for example, Tris-HCl optionally containing sodium chloride and the TNF α INH eluted with an acidic (pH 3.5) glycine buffer. The eluted fractions should immediately be balanced 25 to pH 7.0 by the addition of, for example, Tris base.

The active pooled fractions are preferably lyophilised prior to the final purification step of reverse-phase FPLC (fast protein liquid

chromatography). Prior to its application to the FPLC column, the lyophilised TNF α INH fraction should be buffered with an appropriate buffer such as trifluoroacetic acid (TFA) (Fulka, Buchs, Switzerland), heptafluorobutyric acid (HFBA) or acetic acid. Elution of the TNF α INH from the FPLC column may be carried out using conventional techniques, for example, made up with a suitable buffer as previously described, optionally containing an alcohol, e.g. in stance, N-propanol.

The eluted fraction should immediately be buffered with, for example, ammonium bicarbonate and lyophilised. The TNF α INH is now in a substantially homogenous form suitable for further assaying of biological activity and for preparation in an appropriate form for therapeutic use.

There is thus provided as a further or alternative aspect of the invention a protein which selectively inhibits TNF α -mediated activity which is substantially identical to that obtained in the above process.

The ability to purify TNF α INH of the present invention to homogeneity has enabled the sequencing of the N-terminal portion of this protein molecule. This sequence will assist in the cloning of a gene coding for the TNF α INH thus enabling the production of large quantities of TNF α INH in pure form for further biological investigation and eventually for therapeutic testing and use.

Samples of the homogeneous TNF α INH of the present invention can be sequenced by conventional techniques, for example, using a commercially available automated sequencer employing either ninhydrin or gas phase detection. The first 17 residues of the amino terminal

portion of the human TNF α INH as herein defined comprises the following sequence :

Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile
(identified using an automated sequencer, Model 477A from Applied
5 Biosystems).

It is further believed that the next three amino acids provide a glycosylation site and that the sequence thus continues Asn-Ser-Thr-Lys.

It will be appreciated that one potential method of providing
10 large quantities of TNF α INH in pure form is through recombinant DNA techniques which are well known in the art. However, the successful use of such techniques requires not only that the natural and recombinant TNF α INH or its activity be accurately measured, but also that both the natural and recombinant product be purifiable to
15 homogeneity.

According to yet another aspect of the present invention, there is provided a process for producing a tumour necrosis factor inhibitor of the invention or a derivative thereof by the expression of a DNA sequence encoding for such an inhibitor in a suitable transformed
20 host. Such a process involves the culturing of a host transformed with recombinant DNA molecules comprising DNA sequences encoding the inhibitor which have been inserted into a suitable vector.

Suitable eukaryotic and prokaryotic hosts may be, for example, strains of bacteria, yeasts, other fungi, and animal cells (including
25 insect cells) and plant cells in tissue. Particularly preferred host

cells are yeast cells, E. coli cells and animal cells.

Expression of a protein having tumour necrosis factor inhibitor activity is achieved by culture of the transformed host cells in a suitable growth medium. Normally such a medium will contain a source of nitrogen such as ammonium sulphate, a source of carbon and energy such as glucose or glycerol, trace elements and factors essential to growth of the particular host cells. The precise culture conditions will be dependent upon the chosen host; thus, for example, in the case of E. coli submerged aerobic fermentation is preferred, preferably at about 37°C.

In addition, expression may be induced, for example, by the addition of an inducer or the use of inducing conditions for the promoter system being used in the expression vector.

Depending upon the host, the TNF α inhibitor may be produced as granular inclusion bodies which can be recovered, after cell lysis, by differential centrifugation; these can be solubilised by conventional methods and purified by the methods described herein for purification of urinary TNF α INH. Alternatively, the TNF α inhibitor may be in solution in the cytosol, secreted into the periplasmic space or conveniently secreted into the culture medium.

The host cells are transformed by recombinant DNA molecules which comprise a DNA sequence encoding for a TNF α inhibitor which has been inserted into an expression vector.

Such expression vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV-40 and known bacterial plasmids, for example, "natural" plasmids such as ColEl, pSC101 or pRSF2124 and

phage DNAs, or "artificial" plasmids (constructed in vitro) such as pBR322, pMB9 or pAT153. Phage DNAs include, for example, the numerous derivatives of phage lambda and other DNA phages, for example M13, and other filamentous single-stranded DNA phages. Vectors useful in yeasts include the λ plasmid, and those useful in eukaryotic cells such as animal cells include those containing SV-40 adenovirus and retrovirus.

Such expression vectors may also be characterised by at least one expression control sequence which may be operatively linked to the TNF inhibitor DNA sequence such that it controls and regulates the expression of the cloned DNA sequence. Examples of useful expression control sequences include the lac, trp, tac and trc systems, major operator and promoter regions of phage λ (such as the P_L promoter under the control of the thermolabile ts cl857 repressor), the control region of fd coat protein, the glycolytic promoters of yeast (e.g. the promoter for 3-phosphoglycerate kinase), the promoters of yeast acid phosphatase (e.g. Pho 5), the promoters of yeast α -mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus.

In addition, such expression vectors may possess various sites for insertion of the TNF α inhibitor DNA sequences of this invention. These sites are characterised by the specific restriction endonuclease which cleaves them. Such cleavage sites are well recognised by those skilled in the art. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment and its operative linking to an expression control sequence, is determined by a variety of factors including the number of sites susceptible to a

given restriction enzyme, the size of the protein to be expressed, contamination or binding of the protein to be expressed by host cell proteins which may be difficult to remove during purification, the location of start/stop codons, and other factors recognised by those skilled in the art. Thus the choice of a vector and insertion site for a DNA sequence is determined by a balance of these factors, not all selections being equally effective for a given case.

Likewise, not all host/vector combinations will function with equal efficiency in expressing the DNA sequences of this invention.

The selection is made, depending upon a variety of factors including compatibility of the host and vector, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, or any necessary post-expression modifications of the desired protein.

The DNA sequences of the invention which can express code for proteins with TNF α inhibitor activity may be isolated by screening various DNA libraries for such DNA sequences using a series of DNA probes. The DNA probes may be prepared from the purified natural protein which is used as a source of amino acid sequence data. The purified natural protein may be prepared, for example, from febrile human urine as described above. Degenerate DNA sequences coding for various portions and fragments of the amino acid sequence, e.g. in combination with Latch probes, are used to design the DNA probes.

Thus, various DNA libraries are screened for DNA sequences coding for the TNF α inhibitors of the invention. Such libraries include chromosomal gene banks and cDNA or DNA libraries prepared from cell lines or tissue that are demonstrated to produce TNF α .

inhibitors, such as alveolar macrophages or liver tissue. Screening may be by direct immune expression, for example in λ gt11 or similar systems, or, in the case where a TNF α INH producing cell is identified, by identification of TNF α INH specific mRNA by direct 5 expression in Xenopus oocytes.

A variety of conventional cloning and selection techniques may be used to locate and identify DNA sequences that encode or express in an appropriate eukaryotic or prokaryotic host for the TNF α inhibitors of this invention. These selected DNA sequences may 10 themselves be used as probes to select other DNA sequences coding for TNF α inhibitors or may be used in appropriate recombinant DNA molecules to transform appropriate eukaryotic or prokaryotic hosts for the production of TNF α INH encoded by them.

The invention includes within its scope single and double 15 stranded DNA sequences encoding for TNF α INHs, vectors containing such sequences suitable for transformation of a host organism and host cells transformed with such DNA sequences.

According to a further aspect of the present invention we provide a protein with selective TNF α inhibitor activity produced by 20 expression of a host transformed with a DNA sequence encoding for such a TNF α inhibitor protein. TNF inhibitors of the invention which are prepared by the expression of a DNA sequence encoding such inhibitors in a transformed host will thus be identical to the sequence of native TNF α INH or contain one or more deletions, substitutions, insertions, 25 inversions or additions of allelic origin or otherwise, the resulting sequence will have at least 80% and preferably 90% homology with the sequence of native TNF α INH and retain essentially the same biological

properties. In particular, a TNF inhibitor of the invention may include an N-terminal methionine. Also, for example, the DNA sequence of the invention coding for TNF α INH may be fused in an expression vector to a portion of a DNA sequence coding for a eukaryotic or prokaryotic polypeptide to assist the expression of the TNF α INH encoding DNA sequence or aid secretion, maturation or purification of the TNF α INH from the host; the fused polypeptide may be removed intra-or extra-cellularly by known techniques or the INF α INH may be used together with the fused polypeptide.

The TNF α INHs produced by culturing of the eukaryotic and prokaryotic hosts transformed with DNA sequences encoding for TNF α INHs can then be employed, after purification, in the pharmaceutical compositions of this invention.

It will be appreciated that, when produced by animal cells, the TNF α INH of the invention will be a glycoprotein. Prokaryotic expression systems will, however, produce the protein in an unglycosylated state. In addition, the glycosylated protein may be substantially deglycosylated by techniques known in the art, for example, by the use of endoglycosidase enzymes.

The following non-limiting Examples illustrate the invention. All temperatures are in °C and all percentage concentrations are w/v.

TNF α Inhibition Assay

The percentage of TNF α INH activity in the fractions described in the Examples was determined by assuming that the optical density (OD) values from murine L929 cells stimulated by actinomycin D (acti D) corresponded to 100% inhibition, whereas the OD from cells cultured

with actinomycin D and TNF α corresponded to a maximal cell mortality of 0% TNF α inhibition. The TNF α used in the assay was recombinant human TNF α (rhTNF α) produced in E. coli as described by A. Marmenout et. al., "Molecular cloning and expression of human tumour necrosis factor and comparison with mouse tumour necrosis factor", Eur. J. Biochem., 152, p515 (1985). Thus the percentage of TNF α inhibition in the assay of cytotoxicity was calculated according to formula (I)

10 Percentage of TNF α INH activity =

$$15 \quad 100 \times \left[\frac{(OD \text{ with acti D} + \text{rhTNF}\alpha + \text{TNF}\alpha \text{ INH}) - (OD \text{ with acti D} + \text{rhTNF}\alpha)}{(OD \text{ with acti D}) - (OD \text{ with acti D} + \text{rhTNF}\alpha)} \right] \quad (I)$$

Example 1

Purification of Urinary TNF α INH

a) Concentration of Protein from Human Urine

Human urine (15 litres) was freshly obtained from a pool of
20 five patients prior to any treatment. Two of the patients were suffering from small-cell carcinoma, one from malignant histiocytosis, one from polymyocitis and one from sepsis. All were highly febrile ($>38.5^{\circ}\text{C}$) and devoid of urinary infections. The urine was concentrated at 4° on an Amicon ultrafiltration hollow fibre
25 apparatus, with a molecular size cut-off of about 5 kDa.

b) Precipitation of Protein from Human Urine

The concentrated urine pool was saturated with solid ammonium sulphate by adding the sulphate slowly with constant stirring at 4° until an ammonium sulphate concentration of 40% was reached. The precipitate was removed by centrifugation, discarded, and the supernatant adjusted to 80% saturation with addition of further ammonium sulphate. A pellet was obtained by centrifugation which was resuspended in 150 ml of 20 mM sodium phosphate (pH 7.2) and 150 mM sodium chloride. The ammonium sulphate was removed by dialysis at 4° using 10 mM Tris-HCl pH 7.4, 2 mM EDTA and 5 mM benzamidine HCl.

c) Identification of TNF α INH Activity

The semi-purified fraction of Example 1(b) was tested in a cytotoxicity assay with the TNF-susceptible cell-line L929 in the presence of actinomycin D. At a 1:20 dilution of the semi-purified fraction, total inhibition of the cytotoxic effect induced by rhTNF α was observed so that the OD_{570nm} value was identical to that measured in the presence of actinomycin D alone (OD_{570nm} = 1.5).

Furthermore, inhibitory activity was observed in dilutions of the fraction of up to 1:160 on cells (OD_{570nm} = 0.83) whereas the control value of rhTNF α at a final concentration of 0.2 ng/ml measured in the presence of actinomycin D was lower (OD_{570nm} = 0.73), so that 50% of inhibition was observed at a dilution of approximately 1:100 (OD_{570nm} = 1.10). The TNF α INH had no effect on cell viability when tested without actinomycin D.

d) Comparison of the Effect of TNF α INH on TNF α and β Induced Cytotoxicity

A cytotoxicity assay was conducted using the TNF-susceptible cell-line L929 in the presence of actinomycin D using a range of 5 concentration of TNF α or TNF β to induce the cytotoxic effect. The semi-purified fraction from Example 1(b) was tested at 1:20, 1:50 and 1:80 dilutions. Control tests were performed in the absence of the TNF α or TNF β cytokine, and in the absence of inhibitor. The results are shown in Table 1, below, and demonstrate that the inhibitor of the 10 invention does have some inhibitory effect on TNF β mediated cytotoxicity ranging from approximately 50% down to 2% of the TNF α inhibition with increasing TNF β concentration.

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TABLE 1

Final Concentration of TNF (α or β) (pg/ml) Added to Actinomycin-D Treated L929 cells	Form of Cytokine added to cells	Final Dilution of Sephadex S-200 Inhibitory Fraction on L929 cells (OD_{570nm})			
		None	1/20	1/50	1/80
0	0	>1.90	>1.90	>1.90	>1.90
10	α	ND	ND	ND	ND
	β	1.16	1.66	1.46	1.39
20	α	1.30	>1.90	1.72	1.68
	β	1.02	1.51	1.21	1.22
50	α	1.02	>1.90	1.72	1.68
	β	0.65	1.03	0.84	0.68
100	α	0.73	1.78	1.69	1.51
	β	0.37	0.71	0.59	0.52
250	α	0.38	1.70	1.70	1.33
	β	0.24	0.44	0.31	0.24
500	α	0.30	1.52	1.49	1.11
	β	0.17	0.30	0.26	0.18
1 250	α	0.19	1.09	1.10	0.76
	β	0.11	0.13	0.19	0.13
2 500	α	0.06	1.03	0.80	0.47
	β	ND	ND	ND	ND

- 23 -

Example 2

Gel Filtration of Urinary TNF α INH

The semi-purified TNF α INH of Example 1(b) was purified by gel filtration chromatography at 4° on a Sephadex G-200 column (0.9 x 60 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl buffer (pH7.4) containing 100 mM sodium chloride. A sample of the protein fraction (20 mg, 0.8 ml) was applied to the column and eluted with the same buffer at a flow-rate of 5.4 ml/hr. Fractions (1.35 ml) were collected and tested for TNF α INH activity. The TNF α INH activity eluted from the gel in a single peak. The inhibitory activity showed an apparent molecular weight of 40 to 60 kDa (see Figure 1).

Example 3

Chromatofocusing of Urinary TNF α INH

The semi-purified TNF α INH of Example 1(b) was chromatofocused at 4° on a Mono-P pre-packed column (HR 5/20, 5 x 200 mm) (Pharmacia, Uppsala, Sweden) equilibrated in 25 mM Bis-Tris buffer adjusted to pH 7.1 with imidodiacetic acid (Fluka, Buchs, Switzerland). A sample of the protein fraction of Example 1(b) (30 mg) was applied to the column and eluted with a polybuffer 74/imidodiacetic acid at pH 4.0. Column fractions (1 ml) were tested at 1:10 dilution for their effect in the rhTNF α (0.2 ng/ml) cytotoxicity assay in the presence of actinomycin D (1 μ g/ml). The actual pH of each column fraction was determined with a pH meter, the bulk of the TNF α INH activity being contained in the eluted fractions.

between pH 5.5 and 6.1 (see Figure 2). This is equivalent to the pI of the TNF α INH protein.

Example 4

Ion-Exchange Chromatography of Urinary TNF α INH

The semi-purified TNF α INH of Example 1(b) was purified by anion-exchange chromatography at 4° on a DEAE Sephadex column (2.6 x 20cm) (Pharmacia, Uppsala, Sweden) equilibrated in 10mM Tris-HCl buffer pH 8.0, containing 2mM EDTA. Bound material was eluted from the column with the equilibration buffer containing 0.8M sodium chloride. Fractions (8.0ml) were collected, tested for TNF α INH activity and the inhibitory fractions were pooled (160ml) and dialysed against 10mM sodium acetate buffer pH 5.0 (4 x 2 litres).

The TNF α INH was further purified by cation-exchange chromatography at 4° on a Sulphopropyl-Sephadex column (0.8 x 15 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 10mM sodium acetate buffer pH 5.0. Bound material was eluted from the column with the equilibration buffer containing 0.5M sodium chloride. Fractions (7.5ml) were collected, tested for TNF α INH activity and the inhibitory fractions were pooled and concentrated 20-fold on an Amicon ultrafiltration apparatus with a molecular size cut-off of about 10 kDa.

Example 5

Gel Filtration of Urinary TNF α INH

The TNF α INH concentrate from Example 4 was purified by gel filtration chromatography at 4° on a Sephadex G-200 column (2.6 x 100

cm) (Pharmacia, Uppsala, Sweden) equilibrated with 50mM Tris-HCl pH 7.4 buffer containing 100mM sodium chloride. A sample of the protein fraction from Example 4 (200mg) was applied to the column and eluted with the equilibration buffer at a flow-rate of 27ml/hour. Fractions (9.0ml) were collected, tested for TNF α INH activity and the inhibitory fractions were pooled. The column was calibrated with dextran blue (DB), 2000 kDa; bovine serum albumin (BSA), 67 kDa; ovalbumin (OA), 43 kDa; α -chymotrypsinogen-A (α CT), 25 kDa; and ribonuclease A (RNase), 13.5 kDa, as shown in Figure 4.

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Example 6

Affinity Chromatography of Urinary TNF α INH

A TNF α affinity column was prepared by coupling recombinant human TNF α (1.0mg) to Mini Leak Agarose (Kem En Tec, Biotechnology Corp., Denmark) in 0.8M potassium phosphate buffer pH 8.6. The remaining active groups were blocked by incubation in 0.1M ethanolamine-HCl buffer pH 8.5. The gel was washed with 50mM Tris-HCl pH 7.4 buffer containing 100mM sodium chloride (3 x 50ml). A sample of the TNF α INH fractions from Example 5 (15ml) was applied to the column and eluted with a 0.2M glycine-HCl pH 3.5 buffer. Fractions (1.0ml) were collected, immediately adjusted to pH 7.0 by addition of 1M Tris (5 to 40 μ l) and tested for TNF α INH activity.

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Example 7

Reverse Phase FPLC Chromatography of Urinary TNF α INH

The TNF α INH fractions from Example 6 were lyophilised, dissolved in 0.1% trifluoroacetic acid (2.0ml) and loaded onto a ProRPC

reverse-phase FPLC column (5 x 20 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 0.1% trifluoroacetic acid. Bound material was eluted with a 0 to 100% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 0.3ml/minutes. To each fraction (0.75ml) 0.5M ammonium bicarbonate (10µl) was added and the eluted material was lyophilised.

The reverse-phase FPLC chromatography revealed one major peak corresponding to TNF α INH activity. The lyophilised fractions containing this activity were dissolved in 10mM Tris-HCl pH 7.4 buffer containing 2mM EDTA and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using the method described by U. Laemmli *et al.*, *Nature*, 277, p 680 (1970). The TNF α INH was found to elute with a molecular weight of 33 kDa (see Figure 4). Samples run under non-reducing conditions were tested for TNF α INH activity at 1:10 dilution on L929 cells in the presence of 0.15 mg/ml of rhTNF α . The activity directed against rhTNF α migrated with an apparent molecular weight identical to the 33 kDa band on the gel run under reducing conditions.

Example 8

Protein Sequencing of Urinary TNF α INH

The TNF α INH fraction isolated from the reverse-phase FPLC chromatography was concentrated *in vacuo* and spotted onto a conditioned sequencer filter. The protein was analysed with an Applied Biosystems Model 477A protein sequencer. Fractions from the sequencing cycles were evaporated to dryness and resuspended in N,N-diisopropylethylamine acetate and acetonitrile prior to injection into an HPLC column for residue identification.

The first 17 amino acid residues of the N-terminal were identified and have the sequence : Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile. It is further believed that the next three amino acids provide a glycosylation site and that the 5 sequence thus continues Asn-Ser-Thr-Lys. This sequence is not significantly homogeneous to any protein sequence contained in the NBRF Protein Sequence database (November 1988).

Example 9

10 Demonstration that TNF α INH is a Protein

a) Time and Temperature Dependency

The Sephadryl S-200 purified TNF α INH of Example 2, obtained by bulking the tubes of the active fractions, was heated at 56°, 75° and 95°. The TNF α INH activity was measured after 10, 20 and 60 minutes and, by comparison with untreated samples, the percentage of 15 TNF α INH activity was calculated according to formula (I). The results shown in Table 2 below demonstrate that the TNF α INH activity decreases in a time- and temperature-dependent manner.

Heat inactivation			
	Temperature (°C)	Time (min)	Percentage of TNF α INH activity
5	56	10	100
		20	100
		60	93
	75	10	60
		20	26
		60	15
	95	10	27
		20	10
		60	13

b) Susceptibility to Trypsin Digestion

Trypsin-(500 μ g) (Sigma, St. Louis, MO) in 0.2M Tris-HCl buffer
15 (pH 8.0) containing 1mM calcium chloride was added to the pooled fractions of Sephadryl S-200 purified urinary TNF α INH of Example 2 and incubated at 37°C for 4 hours. Another measure of trypsin (500 μ g) was added and digestion continued for a further 20 hours, at which time the reaction was terminated by addition of soybean trypsin inhibitor (2mg) (Sigma, St. Louis, MO). The percentage of TNF α INH inhibitory activity of the trypsin digest and the control was determined at a 1:20 final dilution of the pool of bulked fractions on L929 cells stimulated by rhTNF α in the presence of actinomycin D, according to formula (I). The results are shown in Table 3 below.

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Trypsin inactivation			
	Conditions	Percentage of TNF α INH activity	OD 570nm
5	Buffer alone	0	0.71
	Trypsin + soybean trypsin inhibitor in buffer	0	0.70
10	Partially purified Sephadex S-200 urine	61	1.46
	Partially purified Sephadex S-200 urine digested by trypsin	23	1.03

c) Treatment with Urea

The Sephadryl S-200 purified TNF α INH of Example 2 was adjusted to 2M urea and extensively dialysed at 4° against phosphate buffered saline (PBS) containing 2M urea. Dialysis against PBS was 15 repeated prior to the bioassay. TNF α INH activity was found to be unaffected which indicates that inhibitory activity is not mediated by a molecule of low molecular weight bound to a protein.

Example 10

Demonstration of Competitive Inhibition

The Sephadryl S-200 purified TNF α INH of Example 2 was tested 20 at a 1:10 dilution against increasing amounts of rhTNF α on L929 cells. An inverse correlation between the amount of rhTNF α present in the assay and the degree of inhibition was observed (see Figure 3). Thus, the inhibitory activity is competitively overcome by increasing concentrations of rhTNF α .

Example 11

Inhibition of TNF α -Mediated PGE₂ Production by Dermal Fibroblasts

Human dermal fibroblasts were seeded at a concentration of 2.0 x 10⁴ cells/well and cultured for 48 hours. Cells were then stimulated with DMEM buffer supplemented with 10% FCS as a control. Cells were also stimulated with rhTNF α at concentrations ranging from 0.5 to 5mg/ml, and the effect of the TNF α INH from Example 5 was studied at three dilutions (1:20, 1:50 and 1:80) in the above buffer. After 72 hours of incubation, PGE₂ production was measured in the supernatants by radioimmunoassay using a PGE₂ antiserum [see J M Dayer *et al.*, *J. Clin. Invest.*, 67, p1386 (1979)].

The results are shown in Table 4, below, and demonstrate that the ability of rhTNF α to stimulate PGE₂ production by dermal fibroblasts was inhibited by the addition of TNF α INH at all three dilutions. At 1:80 dilution of TNF α INH the inhibitory activity was partially overcome by increasing rhTNF α concentrations.

Concentration of rhTNF on human fibroblasts (pg/ml)	PGE ₂ production by human dermal fibroblasts (ng/ml)			
	none	1:80	1:50	1:20
0	50.6 ± 7.4	88.8 ± 5.6	103.0 ± 8.9	111.0 ± 9.4
500	160.0 ± 14.1	126.0 ± 9.3	115.9 ± 6.6	113.9 ± 7.1
2,000	331.7 ± 28.4	217.2 ± 10.7	156.7 ± 10.7	115.3 ± 21.3
5,000	381.7 ± 19.6	257.2 ± 13.7	253.1 ± 21.2	221.6 ± 16.0

Three different experiments were carried out with the same strain of fibroblasts. Buffer or TNF α INH was incubated at various dilutions in the presence or absence of various concentrations of rhTNF α . PGE₂ production by cultured human dermal fibroblasts was measured after three days. Values represent triplicate means of the three cultures ± SEM (N=3).